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RESEARCH PAPER

The novel, potent and highly selective 5-HT$_4$ receptor agonist YH12852 significantly improves both upper and lower gastrointestinal motility

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BACKGROUND AND PURPOSE

5-HT$_4$ receptor agonists have been shown to be effective at treating various gastrointestinal tract disorders. However, a lack of selectivity against off-targets has been a limiting factor for their clinical use.

EXPERIMENTAL APPROACH

The binding affinity and selectivity of YH12852 for human 5-HT$_{4(a)}$ receptor in CHO-K1 cells were evaluated using radioligand binding assays, and agonistic activity was assessed using a β-lactamase reporter system. Contractile activity and propulsive motility were measured in the guinea pig isolated distal colon. Its prokinetic effect on the gastrointestinal tract was evaluated in guinea pigs, dogs and monkeys. Its tissue distribution was evaluated in rats.

KEY RESULTS

YH12852 exhibited high affinity and potency for human recombinant 5-HT$_{4(a)}$ receptor with high selectivity over other 5-HT and non-5-HT receptors, ion channels, enzymes and transporters. YH12852 induced contractions and increased propulsive motility in guinea pig isolated colon. These effects were abolished by the 5-HT$_4$ receptor antagonist GR113808. YH12852 increased defecation more effectively than prucalopride in guinea pigs and dogs and improved gastric emptying more effectively than mosapride in guinea pigs, dogs and monkeys. YH12852 was highly distributed to the gastrointestinal tract as the target organ.

CONCLUSION AND IMPLICATIONS

The high *in vitro* potency and selectivity of YH12852 for 5-HT$_4$ receptor translated into potent *in vivo* efficacy with good tolerability. YH12852 significantly improved both upper and lower bowel motility in the animal models tested and has the potential to address considerable unmet needs in patients with functional constipation, gastroparesis or both.

Abbreviations

FC, functional constipation; FPO, faecal pellet output; GI, gastrointestinal; GIMM, gastrointestinal motility monitoring system; GMC, giant migrating contraction; IA, intrinsic activity; IBS-c, constipation-predominant irritable bowel syndrome; LMMP, longitudinal muscle myenteric plexus

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Introduction

5-HT (serotonin), although not essential, is an important modulator of gut motility and mostly found in the gastrointestinal (GI) tract (Kim and Camilleri, 2000; Keating and Spencer, 2010; Spencer *et al.,* 2011; Heredia *et al.,* 2013). The action of 5-HT is mediated through seven subtypes of **5-HT receptor**, and among the five 5-HT receptor subtypes (5-HT_1, 5-HT_2, 5-HT_3, **5-HT_4** and 5-HT_7 receptors) expressed in the gut (Hoyer *et al.,* 2002), the 5-HT_4 receptor subtype is one of the most intensively studied with regard to gastrointestinal function. The 5-HT_4 receptor is a GPCR and stimulates adenylyl cyclase, increasing cAMP and protein kinase upon its activation (Dumuis *et al.,* 1989; Markstein *et al.,* 1999; Masson *et al.,* 2012). 5-HT_4 receptor agonists simultaneously facilitate the activity of inhibitory nitrergic neurons, inducing nitric oxide release to counteract contraction, and myenteric excitatory cholinergic neurons, enhancing the release of acetylcholine and smooth muscle contraction, which in turn promote motility in the GI tract (Cellek *et al.,* 2006; Mawe and Hoffman, 2013). Evidence shows that 5-HT_4 receptor agonists can act at receptors expressed by enterochromaffin cells, goblet cells and enterocytes of intestinal mucosa, resulting in 5-HT release, mucus release and chloride secretion, respectively, each of which can promote colonic transit (Hoffman *et al.,* 2012). In addition to their prokinetic effects, 5-HT_4 agonists have been reported to reduce visceral hypersensitivity in animals and patients with irritable bowel syndrome (IBS) (Hoffman *et al.,* 2012; Lyubashina *et al.,* 2016).

Consistent with the effects described above, the earliest 5-HT_4 agonists such as **cisapride**, **mosapride** and **tegaserod** were shown to be effective treatments of various functional GI motility disorders including constipation, constipation-predominant IBS (IBS-c), functional dyspepsia and gastroparesis (Deruyttere *et al.,* 1987; Müller-Lissner, 1987; Johanson *et al.,* 2004; McLaughlin and Houghton, 2006; Curran and Robinson, 2008). However, mosapride is only available for upper GI disorders in some Asian markets, while cisapride was withdrawn from the market following reports of cardiovascular side effects due to the relative poor selectivity over human *ether-à-go-go*-related gene potassium channels (**K_v11.1**, hERG channel) (Mohammad *et al.,* 1997; Manabe *et al.,* 2010). Tegaserod was also removed from the market due to the higher rate of cardiovascular events in patients receiving tegaserod over placebo in clinical trials, although a later large epidemiological study found no association between tegaserod and adverse cardiovascular events (Anderson *et al.,* 2009; Tack *et al.,* 2012).

On the basis of the belief that a highly selective 5-HT_4 receptor agonist will provide robust efficacy with improved safety, newer 5-HT_4 receptor agonists such as **prucalopride**, **velusetrag** and naronapride have been developed. These drugs have been demonstrated to be clinically effective especially in patients with chronic constipation (Shin *et al.,* 2014), and no major cardiovascular safety issues have been reported (Manabe *et al.,* 2010; Jiang *et al.,* 2015). Nevertheless, prucalopride is the only selective and efficacious 5-HT_4 agonist in the current market and is only available in certain parts of the world. A significant unmet need therefore exists for new selective 5-HT_4 receptor agonists to expand the available therapeutic options for GI disorders.

In this study, the *in vitro* activity and *in vivo* efficacy of the novel 5-HT_4 receptor agonist YH12852 was investigated. *In vitro* assays were used to characterize its potency, functional activity and selectivity for the 5-HT_4 receptor over a panel of other 5-HT subtypes and non-5-HT receptors. *In vivo* animal studies in rats, guinea pigs, dogs and monkeys evaluated the effects of YH12852 on both upper and lower GI motility. The 5-HT_4 receptor agonists mosapride and prucalopride were used as reference compounds for upper and lower GI motility respectively.

Methods

Animals

All animal study protocols were reviewed and approved by the applicable Institutional Animal Care and Use Committee based on international guidelines. The number of animals used in each study was considered the minimum required to achieve the study objectives, based on regulatory requirements, statistical power and/or availability of historical data. The experimental procedures used in the work described in this article were as humane as possible. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Hartley guinea pigs. The guinea pig is one of the most widely used animal species for the evaluation of 5-HT_4 receptor agonists and easy to operate. Furthermore, the pharmacological properties and distribution of 5-HT_4 receptors in the guinea pigs are similar to those of humans (Sakurai-Yamashita *et al.,* 1999; Takada *et al.,* 1999; Vickery *et al.,* 2007), which enhances the justification for the selection of guinea pigs for these experiments.

Male Hartley guinea pigs were purchased from Orientbio Inc. (Republic of Korea) and housed in cages with free access to reverse-osmosis filtered and UV-sterilized water and food, which passed the radiation test and purchased from Agribrands Purina Korea Inc. (Republic of Korea) under controlled conditions with 12 h-light–dark cycle (Yuhan R&D Institute, Republic of Korea). In the functional 5-HT_4 receptor activity study, which was conducted by a contract research organization (CEREP, Paris, France), guinea pigs were supplied from Charles River with a weight range of 350–400 g.

Guinea pigs were acclimatized to the conditons for at least 5 days before use in each experiment and randomized into groups based on their weight profiles (350–450 g) on the day of experiment for the gastric emptying rate assay, so that mean values of each group were similar. In the faecal pellet output assays, guinea pigs were randomized into groups based on the number of their cumulative faecal pellet output for 6 h and body weight (250–450 g), observed on the day before, so that the mean values of each group were similar.

In the functional 5-HT_4 receptor activity study in the guinea pig isolated distal colon longitudinal muscle myenteric plexus preparation (LMMP), a concentration–response curve was obtained using five tissue preparations for each compound (n = 5 for each group). For the *in vitro* propulsive motility study in guinea pig isolated distal colon, to evaluate the concentration–response curve of YH12852, nine

tissue preparations were exposed to five concentrations of YH12852 (0.01–100 nM) in ascending order (n = 9 for each concentration), except n = 8 for YH12852 0.01 nM concentration because the first application was accidently started with a one step higher concentration (0.1 nM). In the case of YH12852 10 nM (n = 7) or YH12852 100 nM (n = 5), the group sizes are smaller than planned because some tissue preparations were determined to be not suitable for use further for these final-step concentrations due to the reduced responsiveness of tissues. In a separate experiment, to evaluate the effect of pretreatment with a 5-HT_4 receptor antagonist on the prokinetic effect of YH12852 using the same model system, seven colon tissue preparations were used (n = 7 for all groups). In the faecal pellet output assays, a total of five experiments with n = 8 per group was conducted to evaluate the effects of various doses of YH12852, while a vehicle-treated group and prucalopride-treated group were always included in each experiment to ensure the reproducibility of the assay; these served as a negative and positive control group, respectively, and this led to the difference in group size, n = 24 for all groups except vehicle- and prucalopride-treated groups with n = 40. In the same model, two experiments with n = 10 per group were performed to evaluate the dose–response curve of prucalopride and resulted in n = 20 for all groups. At the end of each study, guinea pigs were killed by exposure to 100% carbon dioxide.

Beagle dogs. Dogs are a higher-order animal than rodents, and the anatomical and physiological features of their GI tracts are similar to those of humans. Furthermore, they are more suitable for surgery such as force transducer implantation and repeated serial blood collection due to their larger size than rodent species and also widely used for the evaluation of 5-HT_4 receptor agonists (Nguyen *et al.*, 1997; Briejer *et al.*, 2001; Yamamoto *et al.*, 2009).

Beagle dogs were purchased from Beijing Marshall Biotechnology Co. (China). All dogs were housed in stainless steel cages under controlled conditions with 12 h light–dark cycle (Yuhan R&D Institute, Republic of Korea) and acclimatized for at least 1 month before use in the studies. Animal food was purchased from Purina Korea Inc. and provided to dogs twice daily at approximately 9:00 and 17:00 h with free access to reverse osmosis-filtered and UV-sterilized water through an in-house automatic water supply system. In the study for giant migrating complex (GMC), female dogs were used and the surgery needed to implant transducers for detecting GMC was performed under general anaesthesia in aseptic conditions. Strain gauge force transducers were calibrated and sterilized with ethylene oxide gas before implantation. After the dogs had been anaesthetized with acepromazine (0.02 mg·kg^{-1} i.v.) and ketamine (2 mg·kg^{-1} i.v.), an endotracheal tube was inserted into the trachea with the aid of a laryngoscope to maintain an open airway during the surgery. As an inhaled anaesthetic agent, isoflurane was administered and the extent of anaesthesia was monitored by observing heart rate, body temperature and blood oxygen saturation. The midline of the abdomen was incised to expose the colon, and three force transducers were sutured on the serosal side of the colon in the circular direction at the same interval, with placements in the proximal, middle and distal region of the colon. The neck of each gauge was also sutured on the serosal side of the colon to secure the implantation. The wires of the gauges were guided through a subcutaneous tunnel from the abdomen to the midpoint between the scapulae in the right costal flank made by a stab with a long trocar. After surgery, dogs were administered meloxicam (0.1 mg·kg^{-1} p.o. as oral suspension) and tramadol (4 mg·kg^{-1} p.o. twice a day as oral capsule) daily for at least 1 week and single subcutaneous administration of cefovecin sodium (8 mg·kg^{-1}) as an antibiotic, and the incision sites were cleaned with 2% povidone-iodine solution and dressed daily for at least 1 week to avoid infection. For a full recovery from the surgery, the dogs received at least 2 weeks of post-operative care before the experiments began. Their body weights ranged from 5 to 8 kg at the time of test article administration, and the wires were soldered to the connector and protected by a dog jacket (PJ-D02, Star Medical Inc., Arakawa-ku, Tokyo, Japan). At completion of the study period, all the animals used in the GMC study were killed by an i.v. overdose of pentobarbital (≥8 mL at the concentration of 50 mg·mL^{-1}) and ketamine (≥5 mL at the concentration of 50 mg·mL^{-1}) *via* the cephalic vein. For studying gastric emptying rate, male dogs weighing 10–12 kg at the time of test article administration were used, and all of them were returned to the colony after the study.

In the experiments to evaluate colonic motility, the difference in group size was due to the force transducer becoming detached in some animals. In the gastric emptying assays, some dogs were excluded from the experiments after clonidine injection due to vomiting and this resulted in different group sizes.

Cynomolgus monkeys. The cynomolgus monkey was selected due to the similarity of their digestive tract with that of humans. The number of animals used in the study was considered the minimum required to achieve the study objectives, based on regulatory requirements, statistical power and/or availability of historical data and determined as four monkeys per group in a 4 × 4 Latin crossover design.

Male cynomolgus monkeys originally received from Guangxi Guidong Quadrumana Development & Laboratory Co., Ltd (China) were transferred from a CiToxLAB monkey colony and housed individually in stainless steel monkey cages, due to the nature of this study which does not permit group housing (monitoring of clinical signs and the risk of aggression) under controlled conditions (CiToxLAB North America, a contract research organization, Quebec, Canada). Monkeys were provided with a standard certified commercial chow (Harlan Teklad Certified Hi-Fiber Primate Diet #7195C) twice daily (10 cookies morning and evening) and municipal tap water (which had been exposed to UV light and purified by reverse osmosis) *ad libitum via* an automatic watering system. Treats or fresh fruits/vegetables were also provided as part of the animal enrichment programme. As the animals were already acclimatized to the laboratory environment, at least 7 days were allowed between transfer and the start of treatment. Food was removed at least 16 h before each treatment and provided after the end of the dosing and monitoring. The dose volume of radio-opaque meal [mixture of mashed banana and visipaque (iodixanol 320 mg·mL^{-1}) in a 2:1 ratio] was 10 mL·kg^{-1} administered by oral gavage approximately 60 min after dosing. At the onset of dosing, the age of the animals ranged from 4.5 to 6 years and the body weights ranged from 5.4 to 8.4 kg. At completion of the study

period, all surviving animals were returned to the CiToxLAB North America animal colony.

Sprague Dawley rats. Sprague Dawley rats in the tissue distribution study were purchased from SLAC Laboratory Animal Co., Ltd., China, and used by Wuxi AppTec Co., Ltd., Shanghai, China (a contract research organization). Their body weights ranged from 150 to 280 g, and ages ranged from 7 to 9 weeks on the day of treatment initiation. The rats were acclimatized at Wuxi AppTec for at least 3 days prior to the dosing and housed in polycarbonate solid-bottomed cages with hardwood chip bedding. The rats were fed *ad libitum* with certified rodent diet (catalogue # M-01F, SLAC Laboratory Animal Co., Ltd., Shanghai, China) under a controlled environment. The rats were fasted overnight at least 8 h prior to dosing, and the food returned approximately 4 h after dosing. Water was provided *ad libitum via* water bottles. The water was analysed each quarter by Shanghai Pony Test Technical Co., Ltd. for specific microbes and contaminants including total dissolved solids, inorganic matters, total chlorinated organic chemicals and heavy metal. At the scheduled time for tissue collection, the rats were killed by CO_2 inhalation.

Receptor binding studies

Tissue and cell membrane preparations for radioligand binding. 5-$HT_{4(a)}$ receptor membranes were prepared using GeneBLAzer HTR4-CRE-bla CHO-K1 cells stably expressing human 5-$HT_{4(a)}$ receptor splice variant. The procedures for membrane preparations have been described previously (Mialet *et al.*, 2000). The cells were cultured at 37°C under 5% CO_2 atmosphere in DMEM supplemented with 10% FBS, 25 mM HEPES (pH 7.4), 600 μg·mL^{-1} hygromycin, 0.1 mM non-essential amino acids, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin. Cells were grown to 80% confluence and collected by centrifugation at 190× *g* for 5 min. For the membrane preparation, cell pellets were resuspended in ice-cold 50 mM HEPES buffer (pH 7.4). They were then disrupted by homogenization with a sonicator in ice-cold HEPES buffer (50 mM, pH 7.4). Lysates were centrifuged at 1200× *g* for 5 min at 4°C to remove nuclei and intact cells. The supernatant was then ultracentrifuged at 40 000× *g* for 20 min, and the pellet was resuspended in 50 mM HEPES buffer (pH 7.4). The lysate was further centrifuged at 40 000× *g* for 20 min, and the pellet was resuspended in 50 mM HEPES buffer (pH 7.4), before protein concentrations were determined using a bicinchoninic acid protein assay kit and stored at −80°C.

5-HT_4 receptor binding affinity

Radioligand binding assays were performed in 96-well polypropylene assay plates. The membranes were incubated with [^{3}H]GR113808 in 50 mM HEPES buffer (pH 7.4), containing 0.1% BSA. Each test compound was dissolved in DMSO at 20 mM concentration as a stock solution. The stock solution was diluted to 1 μM with a buffer (50 mM HEPES at pH 7.4 containing 0.1% BSA) and then serially diluted up to the final intended concentrations with the same buffer. Non-specific radioligand binding was assessed using **GR113808** (1 μM). Competition binding studies were conducted using concentrations between 5 pM and 1 nM of YH12852 and a fixed concentration of 0.1 nM [^{3}H]-GR113808 in HEPES buffer containing 0.1% BSA (pH 7.4). Assay plates were incubated for 60 min at 25°C before the binding reaction was terminated by rapid filtration over GF/B filter plates (Perkin Elmer, Shelton, CT, USA) pre-soaked in 0.3% polyethyleneimine. The filter plates were washed three times with filtration buffer (ice-cold 50 mM HEPES buffer pH 7.4) to remove unbound radioactivity. Plates were then dried and the bound radioactivity quantifed by liquid scintillation spectroscopy in a Microscint-20 (PerkinElmer) using a TopCount Scintillation Counter (Packard BioScience, Meriden, CT, USA). Binding data were analysed by non-linear regression analysis using GraphPad Prism™ software (GraphPad Software, Inc., San Diego, CA, USA). pKi (negative decadic logarithm of K_i) values for the test compounds were calculated from the bestfit IC_{50} values and the K_d value of the radioligand using the Cheng–Prusoff equation (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1 + [L]/K_d)$ where [L] = radioligand concentration.

5-HT_4 receptor agonist activity

Functional 5-HT_4 receptor agonist activity was assessed using GeneBLAzer® CRE-bla CHO-K1 cells stably transfected with the human 5-$HT_{4(a)}$ receptor gene, and a β-lactamase reporter, as described previously (Kunapuli *et al.*, 2003). The cells were cultured at 37°C in an atmosphere of 5% CO_2 in DMEM supplemented with 10% FBS, 25 mM HEPES (pH 7.4), 600 μg·mL^{-1} hygromycin B, 0.1 mM non-essential amino acids, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin. Cells were passaged three times per week, at less than 80% confluence. One day before treatment with the test compounds, the cells were collected using 0.5% trypsin/EDTA and then diluted in DMEM supplemented with 1% FBS, 25 mM HEPES and 0.1 mM non-essential amino acids to a concentration of 3.125 × 10^5 cells mL^{-1}; 32 μL aliquots of the diluted cells were added to 384-well plates (10^4 cells per well) and then incubated overnight. Following overnight culture, 8 μL of the medium containing 1% DMSO was added to a cell-free control well and a non-stimulating control well. A test compound was dissolved in DMSO at 20 mM as a stock solution, diluted with assay medium to 0.5 μM and then serially diluted to the final intended concentrations with the same assay medium; 8 μL of the respective test compound dilutions (which had been prepared by diluting 100× with media as described above) containing 1% DMSO were then added to the remaining wells. After incubation for 5 h, the wells were treated with substrate solution (8 μL per well) prepared according to the vendor's instructions (Invitrogen, Carlsbad, CA, USA), and then incubated in the dark for an additional 2 h. 5-HT_4 receptor agonism was evaluated on the basis of fluorescence values of the cleavage-products produced by β-lactamase. After excitation to 410 nm using a fluorescence detector (GENios Pro, Tecan Trading AG, Switzerland), the fluorescence values were measured at two emission wavelengths (first: 465 nm; second: 535 nm). Data were analysed on the basis of the ratio of the fluorescence intensities of each well at the respective wavelengths. Each EC_{50} value was calculated by non-linear regression analysis using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA) programme, based on the activity detected for 8 different concentrations of the test

compounds. Potency data were reported as a mean pEC_{50} value (negative decadic logarithm of the effective concentration producing 50% of the maximum response).

5-HT_4 receptor selectivity

Off-target selectivity screening was conducted by a contract research organization (CEREP, Paris, France). Conventional radioligand binding studies were conducted using membranes prepared from cell lines stably expressing the respective target. A test compound was dissolved in DMSO at 10 mM as a stock solution, diluted [100×], [333×] or [1000×] with 100% DMSO, then either added directly or further diluted [10×] or [5×] with distilled water or assay buffer before addition to the assay well. The % inhibition of specific binding by YH12852 at a single concentration (1 μM, in duplicate) was determined. Follow-up competition binding studies were conducted with increasing concentrations of YH12852 to determine K_i values for those targets at which greater than 50% inhibition was observed at the initial test concentration of 1 μM.

Functional 5-HT_4 receptor agonistic activity in guinea pig isolated distal colon

This study was conducted by a contract research organization (CEREP, Paris, France). Segments of guinea pig distal colon with a length of 1 cm were suspended in 20 mL organ baths filled with an oxygenated (95% O_2 and 5% CO_2) and prewarmed, to 37°C, Krebs–Henseleit physiological salt solution. Pyrilamine (1 μM), methysergide (1 μM) and ondansetron (10 μM) were added to block histamine H_1, 5-HT_2 and 5-HT_3 receptors respectively. Colon segments were stretched to a resting tension of 1 g, then allowed to equilibrate for 60 min, during which time they were washed repeatedly and the tension was readjusted. The experiments were carried out using semi-automated isolated organ systems possessing eight organ baths, with multichannel data acquisition. The maximum change in tension induced by each compound concentration was measured. The tissue samples were exposed to a submaximal concentration of the reference agonist 5-HT (0.3 μM) to verify responsiveness and to obtain a control contractile response. Following washings and a return to basal tension, the samples were exposed to ascending concentrations of YH12852. If an agonist-like response (contraction) was obtained, the highest concentration of the compound was tested again in the presence of the 5-HT_4 receptor antagonist GR113808 (0.1 μM) added 30 min previously, to confirm the involvement of the 5-HT_4 receptors in this response.

Propulsive motility in guinea pig isolated colon tissue

The distal colon of the guinea pig, identified as the region of the colon between the hypogastric flexure and the pelvic brim, was removed and placed in ice-cold Krebs buffer (composition in mM: NaCl, 121; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; and glucose 8; aerated with 95% O_2/5% CO_2; Sigma, St Louis, MO, USA). Each 8 cm segment of distal colon was pinned to each end of a 50 mL organ bath lined with Sylgard and continuously perfused with prewarmed oxygenated Krebs buffer at 10 mL·min^{-1}. The final temperature of the organ bath was 37°C. Gastrointestinal motility was monitored using the gastrointestinal motility monitoring system (GIMM; Med-Associates Inc., Saint Albans, VT, USA). Faecal pellets were prepared by drying faecal pellets collected from guinea pigs and painting them with nail polish before the experiment. Briefly, the distal colon was illuminated from below and a digital video camera was used to film the faecal pellet being propelled in the anal direction. The digital movies were saved and analysed at a later time using software designed for the GIMM. After a 15 min equilibration period, a prepared faecal pellet was inserted into the oral end of the colonic segment and allowed to pass spontaneously until it exited the caudal end of segment. Control values for the rate of propulsion were obtained for each preparation by obtaining an average rate of propulsion from five trials before the addition of any drugs. Drugs were prepared at 20 mM concentration in DMSO as a stock solution and then diluted with Krebs buffer to be dissolved in 0.01% DMSO solution (or 0.1% DMSO solution for GR113808) and warmed up to 37°C before use. Pre-warmed vehicle or 5-HT_4 agonist solution was perfused into the orad part of isolated colonic segment using PE-10 tubing, which was inserted through the caudal end and advanced close to the orad end at a rate of 0.25 mL·min^{-1} for 30 s, whereas pretreatment with 5-HT_4 antagonist GR113808 or vehicle was intraluminally applied for 10 min before the perfusion of vehicle or 5-HT_4 agonist solution. Five individual runs were performed for each new drug or concentration change. Each experiment was performed on at least five different colon specimens from at least five different animals. Approximately 5 min was allowed to elapse between runs. The rate of motility of the faecal pellets was analysed by monitoring the time it took for a faecal pellet to traverse 5 cm of the colon. The rates of propulsion were compared between all groups.

Faecal pellet output in guinea pigs

Acclimatized male Hartley guinea pigs weighing 250 to 450 g were divided into several groups, based on their body weight and cumulative faecal pellet output values for 6 h, observed 1 day before the experiment, so that the mean values for each group were similar. On the day of the experiment, faecal pellet output was measured in non-fasted guinea pigs receiving oral administration of vehicle (0.5% methyl cellulose solution), YH12852 or prucalopride. Immediately following dosing, the animals were placed into clean experimental cages for observation and faecal pellet output was recorded for 6 h.

Giant migrating contractions in dogs

Overnight fasted dogs were anaesthetized and implanted with three force transducers in the circular direction with the same anatomical interval (placed in the proximal, middle and distal regions of the colon). The animals then received at least 2 weeks of post-operative care for a full recovery from the surgery before their colonic motility patterns were monitored using a telemetric recording system (GTS-850 system, Star Medical, Tokyo, Japan) in a conscious freely moving state. After 24 h fasting with free access to water, the test article was administered p.o. in the morning of the experiment and the patterns of colonic contraction and behaviour were recorded for 24 h using both the telemetric recording system and video cameras with infrared night vision. Within the

recorded colonic contraction patterns, GMCs were identified and the time points for each GMC after administration of test drug/vehicle were recorded to determine the time taken to the first GMC as the primary endpoint (and GMC frequency as secondary endpoint). A GMC was defined as a single high-amplitude distally propagated contraction with an extended duration (higher than 100 mmHg in amplitude, longer than 20 s in duration).

Gastric emptying rate in guinea pigs

This method was adapted and modified from the procedure described by Scarpignato *et al*. (1980) and Sonia *et al*. (Goineau *et al.,* 2015). It is based on the principle that phenol red is a non-absorbable marker compound. Acclimatized male Hartley guinea pigs weighing 350 to 450 g were divided into several groups by weight to ensure that the mean values of each group were similar. Without fasting, the animals were administered vehicle or various doses of YH12852 (0.1–3 $mg \cdot kg^{-1}$). One hour after administration, 2 mL of phenol red solution in 1.5% methylcellulose was administered intragastrically with an orogastric cannula. Thirty minutes later, the animals were killed and the stomachs were immediately removed after clamping at the oesophageal sphincter and pylorus to prevent leakage of phenol red solution. Each whole stomach, including the stomach contents, was placed in 25 mL 0.1 M NaOH and minced, before being left at room temperature for 1 h. The supernatant was then centrifuged at 1200 *g* for 10 min. The absorbance at 546 nm was measured with a spectrophotometer to calculate the phenol red content remaining in the stomach. The increase in gastric emptying rate (%) compared to the vehicle-treated group was calculated as $(B - A)/B \times 100$, where A = the amount of phenol red remaining in the stomach of each animal in the YH12852-treated group; and B = the average amount of phenol red remaining in the stomach in the vehicle-treated group.

Gastric emptying rate in a canine model of gastroparesis

Ten male beagle dogs, weighing between 9 and 11 kg, were fasted overnight and fed 10 $g \cdot kg^{-1}$ of meal (Adult Gourmet Chicken Entrée Canned Dog Food, Hill's Science Diet) on the morning of the experiment. To induce delayed gastric emptying, clonidine (10 $\mu g \cdot kg^{-1}$) was injected i.v. 15 min before feeding. YH12852 (0.1, 0.3, 1, 2 and 3 $\mu g \cdot kg^{-1}$), mosapride (600, 1000 and 3000 $\mu g \cdot kg^{-1}$) or vehicle (water) were administered p.o. 15 min before the clonidine injection. Five minutes after the meal, acetaminophen solution at a dose of 10 $mg \cdot kg^{-1}$ in 2 mL was administered p.o. by gastric gavage. Blood samples were collected from the cephalic vein every 15 min for a duration of 90 min. The plasma concentration of acetaminophen was measured with a validated LC–MS/MS method (Zou *et al.,* 2008; Gicquel *et al.,* 2013) and used as an indirect marker of gastric emptying rate, based on the fact that acetaminophen is not absorbed through the stomach but quickly absorbed through the proximal small intestine (Wyse *et al.,* 2003). This method has been used successfully to evaluate drug effects on dog gastric emptying (Tanaka *et al.,* 1998; Onoma *et al.,* 2008).

Gastric emptying and small intestinal transit time in monkeys

This study was conducted by a contract research organization (CiToxLAB, Quebec, Canada). Four male cynomolgus monkeys, weighing between 4.5 and 6.4 kg, were fasted overnight and administered vehicle (0.5% methylcellulose solution) or YH12852 *via* stomach gavage. One hour after drug administration, a radio-opaque material mixed with the meal was provided p.o. Immediately following the meal, radiography was taken at regular intervals for 4 h (5 min intervals for the first 1 h and 10 min intervals for the next 3 h). Gastric emptying time and small intestinal transit time were determined by evaluating the location and amount of meal left in the stomach and small intestine.

Tissue distribution study of YH12852 in rats

Tissue distribution of YH12852 in Sprague Dawley rats was assessed by Wuxi AppTec (Shanghi, China). Following p.o. administration of YH12852 in rats at 30 $mg \cdot kg^{-1}$, blood was taken under anaesthesia at 4, 8 and 24 h post-dose (three rats per time point). A total of 10 tissue samples (brain, heart, liver, lung, kidney, spleen, testis, stomach, small intestine and large intestine) were isolated from the killed rats. The tissue samples were washed with ice-cold saline after the contents of the gastrointestinal tract had been removed. The concentration of YH12852 in the plasma and tissue samples was quantified by LC–MS/MS, and the tissue to plasma ratio was calculated.

Data analysis

All studies, except the functional 5-HT_4 receptor activity study in LMMP, were not performed blind. However, we made an effort to be close to the conditions of blinded assays. All the samples were obtained *via* the same procedures and treated in the same way. All data were obtained *via* direct recording or measurement of objective endpoints, so that analysis did not include any subjective evaluation.

The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.,* 2015). Results are expressed as the mean ± SEM except where specified. Data from *in vitro* binding affinity and agonistic activity studies were generated by non-linear regression analysis with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA, Version 5.01) based on the effects of different concentrations of the test compounds. The effective doses that induced 50% of the maximal effect (ED_{50}) and 95% confidence limits for data from *in vivo* studies were also estimated using nonlinear regression in Graphpad Prism. Significance was established between two groups using Student's *t*-test, while ANOVA was used for multiple group comparisons followed by Dunnett's *post hoc* test with GraphPad Prism software. The *post hoc* test was run only if *F* value achieved $P < 0.05$ and there was no significant variance in homogeneity by Levene's test. Differences were considered significant at $P < 0.05$, and significant results are reported with this single level of probability ($P < 0.05$) throughout the article. Meanwhile, statistical analysis was not performed for the studies in which an insufficient

number of animals was used (less than five per group) (e.g. GI transit time study in monkeys).

Reagents and compounds

Standard biochemical and cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA) respectively. **[^{3}H]-GR113808** was purchased from GE Healthcare (Buckinghamshire, UK). GeneBLAzer® CRE-bla CHO-K1 cells were purchased from Invitrogen (Carlsbad, CA, USA). YH12852 was synthesized at Yuhan R&D Institute (Republic of Korea). **GR113808**, prucalopride and tegaserod were purchased from Sigma-Aldrich (St. Louis, MO, USA), Apichem Chemical Technology (Shanghai, China) and Epochem (Shanghai, China) respectively.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Results

5-HT$_4$ receptor binding affinity and cAMP accumulation

YH12852, tegaserod and prucalopride were observed to inhibit [^{3}H]-GR113808 binding to CHO-K1-h5-HT$_{4(a)}$ cell membranes in a concentration-dependent manner. YH12852 showed the highest affinity (pKi = 10.3) for the human recombinant 5-HT$_{4(a)}$ receptor, followed by tegaserod (pKi = 8.5) and prucalopride (pKi = 7.8) (Table 1, Figure 1A). In 5-HT$_4$ receptor agonistic activity assays using CHO-K1 cells stably transfected with the human 5-HT$_{4(a)}$ receptor gene, treatment with YH12852 produced concentration-dependent increases in 5-HT$_{4(a)}$ receptor agonistic activity. YH12852 (pEC$_{50}$ = 11.4) exhibited more potent agonistic activity than both tegaserod (pEC$_{50}$ = 10.6) and prucalopride (pEC$_{50}$ = 9.5) (Table 1, Figure 1B).

Table 1

In vitro data comparison of YH12852, tegaserod and prucalopride

	Human 5-HT$_{4(a)}$ receptor affinity	Human 5-HT$_{4(a)}$ agonistic activity	Contractile activity in guinea pig colonic LMMP	
Compound	pKi (mean ± SEM)	pEC$_{50}$ (mean ± SEM)	pEC$_{50}$ (mean ± SEM)	Intrinsic activity (mean ± SEM)
YH12852	10.3 ± 0.02*,#	11.36 ± 0.05*,#	8.40 ± 0.02*	101 ± 2*
Prucalopride	7.84 ± 0.04	9.48 ± 0.06	7.94 ± 0.03	110 ± 2
Tegaserod	8.49 ± 0.03	10.63 ± 0.05	8.2 (8.0–8.4)[1)]	65 (64–66)[1)]

The binding affinity (pKi, mean values, n = 5) and agonistic activity (pEC$_{50}$, mean values, n = 5) at human 5-HT4$_{(a)}$ receptor for each compound were measured using CHO-K1 cells. The agonistic potency (pEC$_{50}$) and intrinsic activity (% of the 5-HT maximum response) for each compound were also determined using guinea pig isolated colon LMMP (mean values, n = 5). Significantly different from prucalopride (*: $P < 0.05$) or tegaserod data (#: $P < 0.05$). All data are expressed as the mean ± SEM, except an excerpt from the reference literature (Smith *et al.*, 2008)[1)], and these data represent the mean values with 95% confidence intervals in the same manner in the reference.

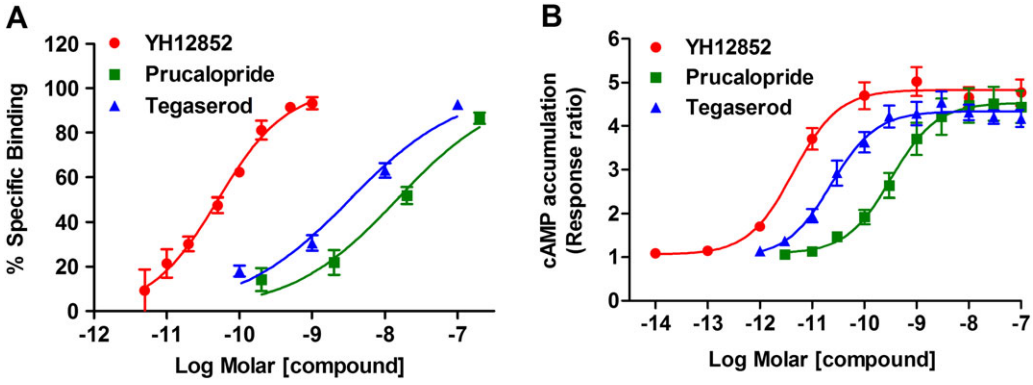

Figure 1

In vitro comparison of YH12852, tegaserod and prucalopride. (A) Competitive inhibition of [^{3}H]GR113808 binding to human 5-HT$_{4(a)}$ receptors, in membranes prepared from CHO-K1 cells stably transfected with the human 5-HT$_{4(a)}$ receptor gene, by YH12852, tegaserod and prucalopride. Data represent mean ± SEM (n = 5). (B) Agonistic activity for the human 5-HT$_4$ receptor was measured using CHO-K1 cells stably expressing 5-HT$_{4(a)}$ receptors with a β-lactamase reporter gene under the control of a cAMP response element. Data represent mean ± SEM (n = 5).

Table 2

Pharmacological profile of YH12852 (5-HT receptors and non-5-HT receptors)

Receptor	Subtype	Radioligand	% inhibition	Ki (nM)
Adenosine	A_1 (human)	[^{3}H]DPCPX	-	>1000
	A_{2A} (human)	[^{3}H]CGS 21680	-	>1000
	A_3 (human)	[^{125}I]AB-MECA	49	n.t.
Adrenoceptors	α_1 (non-selective)	[^{3}H]prazosin	-	>1000
	α_2 (non-selective)	[^{3}H]RX 821002	-	>1000
	β_1 (human)	[^{3}H](-)CGP 12177	38	n.t.
	β_2 (human)	[^{3}H](-)CGP 12177	-	>1000
Angiotensin II	AT_1 (human)	[^{125}I][Sar^1,Ile^8]-AT-II	-	>1000
	AT_2 (human)	[^{125}I]CGP 42112A	-	>1000
Benzodiazepine	BZD (central)	[^{3}H]flunitrazepam	-	>1000
	BZD (peripheral)	[^{3}H]PK 11195	71	430
Bombesin	BB (non-selective)	[^{125}I][Tyr^4]bombesin	-	>1000
Bradykinin	B_2 (human)	[^{3}H]bradykinin	-	>1000
Calcitonin gene-related peptide	CGRP (human)	[^{125}I]hCGRPα	-	>1000
Cannabinoid	CB_1 (human)	[^{3}H]CP 55940	-	>1000
Cholecystokinin	CCK_1 (CCKA) (human)	[^{125}I]CCK-8 s	48	n.t.
	CCK_2 (CCKB) (human)	[^{125}I]CCK-8 s	-	>1000
Dopamine	D_1 (human)	[^{3}H]SCH 23390	85	120
	D_{2S} (human)	[^{3}H]methylspiperone	-	>1000
	D_3 (human)	[^{3}H]methylspiperone	-	>1000
	$D_{4.4}$ (human)	[^{3}H]methylspiperone	-	>1000
	D_5 (human)	[^{3}H]SCH 23390	71	270
Endothelin	ET_A (human)	[^{125}I]endothelin-1	-	>1000
	ET_B (human)	[^{125}I]endothelin-1	-	>1000
GABA	GABA (non-selective)	[^{3}H]GABA	-	>1000
Galanin	GAL_1 (human)	[^{125}I]galanin	-	>1000
	GAL_2 (human)	[^{125}I]galanin	-	>1000
Growth factors	PDGF	[^{125}I]PDGF BB	-	>1000
Chemokines	CXCR2 (IL-8B) (human)	[^{125}I]IL-8	-	>1000
–	CCR1 (human)	[^{125}I]MIP-1α	-	>1000
Cytokines	TNF-α (human)	[^{125}I]TNF-α	-	>1000
Histamine	H_1 (human)	[^{3}H]pyrilamine	-	>1000
	H_2 (human)	[^{125}I]APT	-	>1000
Melanocortin	MC_4 (human)	[^{125}I]NDP-α-MSH	-	>1000
Melatonin	MT_1 (ML1A) (human)	[^{125}I]2-iodomelatonin	-	>1000
Muscarinic	M_1 (human)	[^{3}H]pirenzepine	-	>1000
	M_2 (human)	[^{3}H]AF-DX 384	-	>1000
	M_3 (human)	[^{3}H]4-DAMP	-	>1000
	M_4 (human)	[^{3}H]4-DAMP	-	>1000
	M_5 (human)	[^{3}H]4-DAMP	-	>1000
Neurokinin	NK_1 (human)	[^{125}I]BH-SP	-	>1000
	NK_2 (human)	[^{125}I]NKA	-	>1000
	NK_3 (human)	[^{3}H]SR 142801	-	>1000
Neuropeptide Y	Y_1 (human)	[^{125}I]peptide YY	-	>1000
	Y_2 (human)	[^{125}I]peptide YY	-	>1000
Neurotensin	NTS_1 (human)	[^{125}I]Ty r^3-neurotensin	-	>1000

continues

Table 2
(Continued)

Receptor	Subtype	Radioligand	% inhibition	Ki (nM)
Opioid and opioid-like	δ receptor (human)	[^{3}H]DADLE	-	>1000
	κ receptor	[^{3}H]U 69593	-	>1000
	μ receptor (human)	[^{3}H]DAMGO	-	>1000
	NOP receptor (human)	[^{3}H]nociceptin	-	>1000
Non-steroid nuclear receptors	PPAR γ (human)	[^{3}H]rosiglitazone	-	>1000
Glutamate channels	PCP	[^{3}H]TCP	-	>1000
Prostanoid	EP_2 (human)	[^{3}H]PGE_2	-	>1000
	IP (PGI_2) (human)	[^{3}H]iloprost	-	>1000
Purine	P2X	[^{3}H]α,β-MeATP	-	>1000
	P2Y	[^{3}5S]dATPαS	-	>1000
5-HT	5-HT_{1A} (human)	[^{3}H]8-OH-DPAT	71	62
	5-HT_{1B}	[^{125}I]CYP(+ 30 μM isoproterenol)	-	>1000
	5-HT_{2A} (human)	[^{3}H]ketanserin	-	>1000
	5-HT_{2B} (human)	[^{125}I](±)DOI	97	11
	5-HT_{2C} (human)	[^{3}H]mesulergine	50	340
	5-HT_3 (human)	[^{3}H]BRL 43694	-	>1000
	5-HT_{4e} (human)	[^{3}H]GR113808	100	0.052
	5-HT_{5a} (human)	[^{3}H]LSD	47	530
	5-HT_6 (human)	[^{3}H]LSD	58	320
	5-HT_7 (human)	[^{3}H]LSD	-	>1000
hERG	K_v11.1 (human)	[^{3}H]astemizole	-	>1000
Sigma	σ (non-selective)	[^{3}H]DTG	-	>1000
Somatostatin	SST (non-selective)	[^{125}I]Tyr^{11}-somatostatin-14	-	>1000
Steroid nuclear receptors	GR (human)	[^{3}H]dexamethasone	-	>1000
Vasoactive intestinal	PAC_1 (PACAP) (human)	[^{125}I]$PACAP_{1-27}$	-	>1000
peptide	$VPAC_1$ (VIP1) (human)	[^{125}I]VIP	-	>1000
Vasopressin	V_{1A} (human)	[^{3}H]AVP	-	>1000
Ca^{2+} channels	Ca^{2+} channel (L, verapamil site) (phenylalkylamine)	[^{3}H]D888	-	>1000
K^+ channels	K_V channel	[^{125}I]α-dendrotoxin	-	>1000
	SK_{Ca} channel	[^{125}I]apamin	-	>1000
Na^+ channels	Na^+ channel (site 2)	[^{3}H]batrachotoxinin	-	>1000
GABA channels	Cl^- channel (GABA-gated)	[^{35}S]TBPS	-	>1000
Noradrenaline transporter	NET (human)	[^{3}H]nisoxetine	-	>1000
Dopamine transporter	DAT (human)	[^{3}H]BTCP	-	>1000
5-HT (serotonin) transporter	SERT (human)	[^{3}H]imipramine	-	>1000

The % inhibition of specific binding by YH12852 was determined at the single concentration of 1 μM with a hyphen (-) used to denote cases where the % inhibition was less than 30%. The results are expressed as the % inhibition of control-specific binding (mean values, $n = 2$). Follow-up competition binding studies were conducted with increasing concentrations of YH12852 to determine K_i values for those targets at which greater than 50% inhibition was observed at the initial test concentration of 1 μM, and n.t. stands for not tested.

YH12852 exhibits high 5-HT_4 receptor selectivity

YH12852 exhibited high selectivity for the 5-HT_4 receptor over other 5-HT receptor subtypes and non-5-HT receptors including classical monoamine and peptide receptors, ion channel binding sites, lipid-derived factors and neurotransmitter transporter binding sites (Table 2, assays performed on 80 targets). YH12852 showed low binding affinity for the benzodiazepine receptor, dopamine D_1 and D_5 receptors. No inhibitory value higher than 50% was found in the other binding tests up to 1 μM including for the hERG channel. In competition binding tests on 5-HT receptor

subtypes, YH12852 bound with high affinity to the 5-$HT_{4(e)}$ receptor compared to binding affinity for the 5-HT_{2B}, 5-HT_{1A} and 5-HT_6 receptor. Therefore, YH12852 exhibits high selectivity for the 5-$HT_{4(e)}$ receptor over other 5-HT receptors (including 5-HT_{2B} and 5-HT_{1A}).

YH12852 induces contractions in guinea pig colonic longitudinal muscle/myenteric plexus

YH12852 induced concentration-dependent contractions in guinea pig colon with maximal activity at a concentration of $>\log^{-7}$ M, while the maximal effect of prucalopride was achieved at a concentration of $>\log^{-6}$ M (Figure 2).

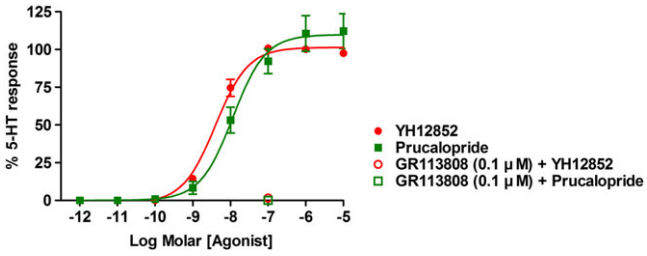

Figure 2

Effect of YH12852 and 5-HT on contractile activity in guinea pig distal colon. Concentration–response curves for YH12852 and prucalopride in guinea pig isolated colonic LMMP preparation. The contractile response to each agonist was calculated as the relative percentage of control response to 5-HT (0.3 μM) in the same tissue and then expressed as mean ± SEM (n = 5 for each group). In the presence of the 5-HT_4 antagonist GR113808 (0.1 μM), the effects of both YH12852 and prucalopride were completely blocked.

Comparison of the mean pEC_{50} values for the compounds (Table 1) indicated a rank order of potency of YH12852 (pEC_{50} = 8.4) > tegaserod (pEC_{50} = 8.2) > prucalopride (pEC_{50} = 7.9). Comparison of the mean EC_{50} values (4 nM vs. 12 nM) indicated that YH12852 was approximately three times more potent than prucalopride with a mean intrinsic activity at 101% of the maximal effect of 5-HT, thus behaving as a full agonist. The maximal effect of prucalopride was 110% that of 5-HT (Table 1). Pretreatment with the selective 5-HT_4 receptor antagonist GR113808 (0.1 μM) completely blocked the effect of both YH12852 and prucalopride.

Intracolonic administration of YH12852 increases propulsive motility in isolated guinea pig colon

Intracolonic administration of YH12852 was observed to cause a concentration-dependent increase in the rate of fecal pellet propulsion in isolated segments of the guinea pig distal colon (Figure 3). In particular, YH12852 at 1 nM caused maximum increase in propulsion velocity by 50.6% compared to the vehicle. Meanwhile, pretreatment of the selective 5-HT_4 receptor antagonist GR113808 100 nM caused a significant decrease in propulsion by 23.4% compared to the vehicle pretreatment, and GR113808 pretreatment completely blocked the increase in propulsion velocity induced by YH12852 1 nM, indicating that the propulsive effect of YH12852 occurs *via* activation of the 5-HT_4 receptor.

YH12852 is superior to prucalopride in enhancing faecal pellet output in guinea pigs

Oral administration of YH12852 significantly increased faecal pellet output (FPO) at 0.1–10 $mg{\cdot}kg^{-1}$ ($P < 0.05$), while the effect on FPO was reduced at 30 $mg{\cdot}kg^{-1}$ showing a

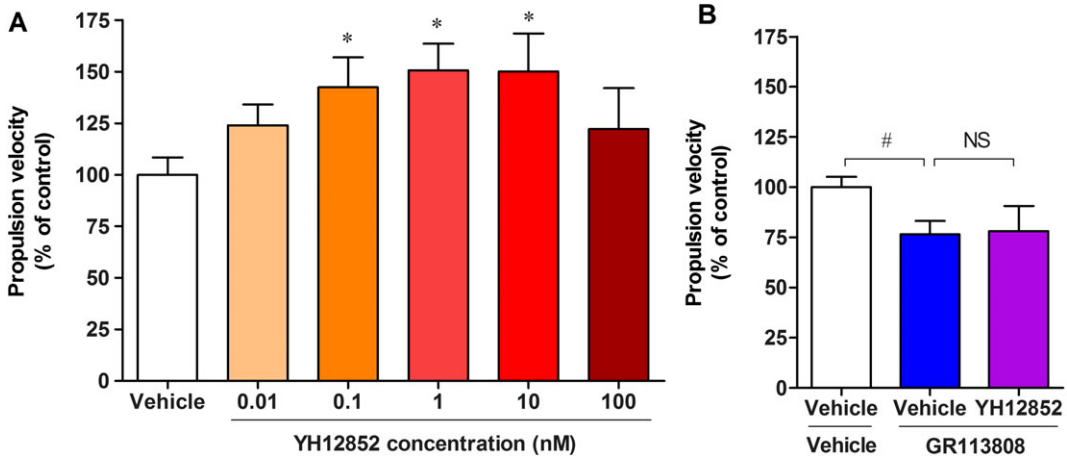

Figure 3

Effect of YH12852 on propulsive motility in guinea pig isolated colon. A gastrointestinal motility monitor was used to record and analyse the propulsive motility in guinea pig colonic segments. (A) Intracolonic administration of YH12852 increases the rate of propulsive motility (n = 9 for each concentration of YH12852 tested, except n = 8 for YH12852 0.01 nM, n = 7 for YH12852 10 nM and n = 5 for YH12852 100 nM concentration). (B) The effect of YH12852 (1 nM) was blocked by the 5-HT_4 receptor antagonist GR113808 (100 nM) (n = 7 for all groups). Results are expressed as mean ± SEM and statistically analysed by one-way ANOVA followed by Dunnett's test (*$P < 0.05$ vs. the control group) or by Student's t-test (#$P < 0.05$ or NS, not significantly different each other).

bell-shaped dose–response (Figure 4). The maximal effect of prucalopride was achieved at the dose of 3 mg·kg^{-1} which was comparable to that of YH12852 at 0.3 mg·kg^{-1} (Figure 4B). The maximum response to YH12852 at 10 mg·kg^{-1} was also approximately twice greater than that of prucalopride at 10 mg·kg^{-1} (Figure 4A).

YH12852 reduces the time to first GMC and increases the number of GMCs in dogs

Oral administration of YH12852 significantly shortened the time to first GMC at doses of 3, 10 and 30 μg·kg^{-1} in a dose-dependent manner ($P < 0.05$) (Figure 5A). YH12852 also elicited a tendency to increase GMC frequency at doses from 3–30 μg·kg^{-1} in a dose-dependent manner, and this effect was statistically significant at 30 μg·kg^{-1} ($P < 0.05$) (Figure 5B).

YH12852 increases gastric emptying rate in guinea pigs

YH12852 significantly improved gastric emptying at all doses, exhibiting a bell-shape dose–response curve. The maximum effective dose of YH12852 was 1.0 mg·kg^{-1} ($P < 0.05$) (Figure 6).

YH12852 recovers delayed gastric emptying in a canine model of clonidine-induced gastroparesis

An i.v. injection of clonidine caused a significant decrease in the maximum concentration (Cmax), the time taken to reach the maximum concentration (Tmax) and the AUC for acetaminophen, indicative of a delay in gastric emptying ($P < 0.05$). Based on the Cmax and AUC values, pretreatment with YH12852 tended to prevent the delayed gastric emptying rate from a dose of 0.1 μg·kg^{-1} in a dose-dependent manner and reached statistical significance at the dose of 1.0 μg·kg^{-1} ($P < 0.05$). Of particular note, administration of 1 μg·kg^{-1} YH12852 fully prevented the clonidine-induced delayed gastric emptying. However, 2 μg·kg^{-1} caused slightly lower levels of recovery, while 3.0 μg·kg^{-1} had no effect. In comparison, mosapride was unable to restore the delayed gastric emptying rate at any of the doses tested (Figure 7).

Administration of YH12852 in non-human primates results in a significant decrease in gastric emptying time and small intestinal transit time

In the preliminary dose range-finding study (n = 2 per group), YH12852 resulted in a substantial decrease in gastric emptying time and small intestinal transit time at all dose levels (0.03–3 mg·kg^{-1}) with maximal response for gastric emptying time at 0.3 mg·kg^{-1} (68 min compared to 235 min for control) and for small intestinal transit time at 0.1 and 0.3 mg·kg^{-1} (38 min for both doses compared to 105 min for control). Based on the preliminary study result, the main study was performed using a 4 × 4 Latin crossover design in four monkeys. Consistent with the preliminary result, YH12852 dramatically shortened gastric emptying time as well as small intestinal transit time (Figure 8).

Tissue distribution of YH12852 in rats

After p.o. administration, YH12852 was widely distributed into tissues and highly distributed in target tissues (stomach, small intestine and large intestine). Mean tissue to plasma ratios (T/P ratio) of YH12852 at 4 h were 147.2, 428.5 and 321.9

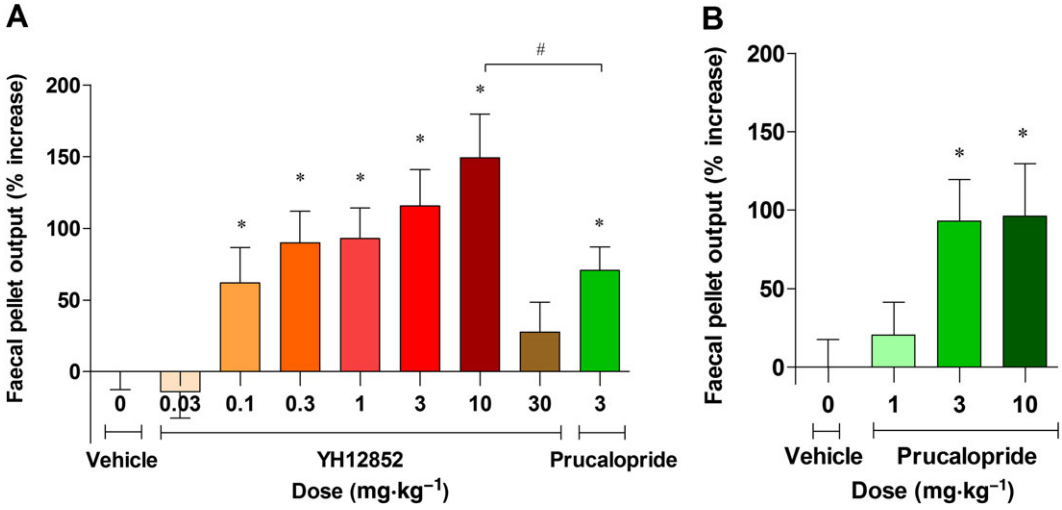

Figure 4

Dose–response effect of (A) YH12852 or (B) prucalopride on faecal pellet output in non-fasted guinea pigs. (A) n = 24 for all groups except vehicle- and prucalopride-treated groups where n = 40, (B) n = 20 for all groups. Results are expressed as mean ± SEM and statistically analysed by one-way ANOVA followed by Dunnett's test (* $P < 0.05$ vs. the vehicle-treated control) or by Student's t-test (# $P < 0.05$, between two groups, YH12852 10 mg·kg^{-1} treated group and prucalopride 3 mg·kg^{-1} treated group, which seemed to show maximal effect of each compound).

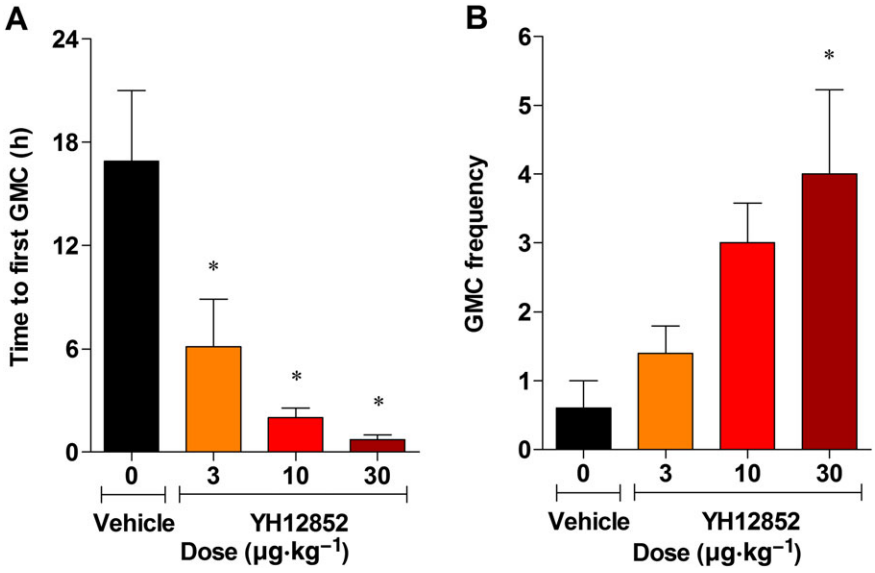

Figure 5

Effect of YH12852 on (A) time to the first GMCs and (B) the total number of GMCs in dogs. For all groups $n = 5$, except $n = 6$ for YH12852 10 μg·kg^{-1} treated group. Results are expressed as mean ± SEM and statistically analysed by one-way ANOVA followed by Dunnett's test (* $P < 0.05$ vs. the vehicle-treated control).

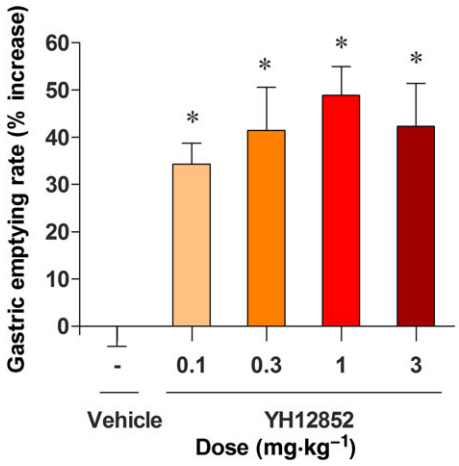

Figure 6

Effect of YH12852 on gastric emptying rate in non-fasted guinea pigs. For all groups, $n = 10$. Results are expressed as mean ± SEM and statistically analysed by one-way ANOVA followed by Dunnett's test (* $P < 0.05$ vs. the vehicle-treated control).

in the stomach, small intestine and large intestine respectively. The T/P ratios for YH12852 at 8 h were 47.1, 56.0 and 1464.4 in the stomach, small intestine and large intestine respectively. The YH12852 concentrations in plasma and the target tissues at 24 h were below the lower limit of quantification. Meanwhile, YH12852 was not detected in the brain at any time points.

Discussion

Functional GI disorders associated with reduced GI motility including functional constipation (FC), IBS-c, functional dyspepsia and gastroparesis together constitute the most common diseases of the digestive tract and significantly affect quality of life for patients (Sanchez and Bercik, 2011; Canavan *et al.,* 2014; Talley and Ford, 2015). Given the fact that functional GI disorders have been defined exclusively based on their symptoms, it is very likely that each disease has diverse aetiological factors and GI dysmotility might only be one of those. However, prokinetic drugs can still be effective therapeutic agents in some clinical conditions either where increased gastric emptying is known to be required or related to disrupted GI motility (Sanger and Alpers, 2008). As with a prokinetic profile, 5-HT$_4$ receptor agonists therefore primarily aim to restore the underlying hypomotility associated with these disorders.

YH12852 is a structurally novel chemical entity based on a 2, 4-diaminopyrimidine scaffold and, based on *in vitro* data, has a higher affinity and potency for human recombinant 5-HT$_{4(a)}$ receptors than tegaserod and prucalopride. YH12852 was also found to be approximately three times more potent than prucalopride and to have full intrinsic activity (IA) almost equal to endogenous 5-HT or prucalopride in the guinea pig colonic LMMP. Pretreatment with the selective 5-HT$_4$ receptor antagonist GR113808 completely blocked the effect of YH12852, confirming its target as the 5-HT$_4$ receptor. According to published data, obtained using a similar model, prucalopride showed the highest IA among the 5-HT$_4$ agonists tested (prucalopride > cisapride >> **TD-8954** >> tegaserod > mosapride) (Beattie *et al.,* 2011). YH12852 also exhibited high selectivity for the human 5-HT$_{4(e)}$ receptor

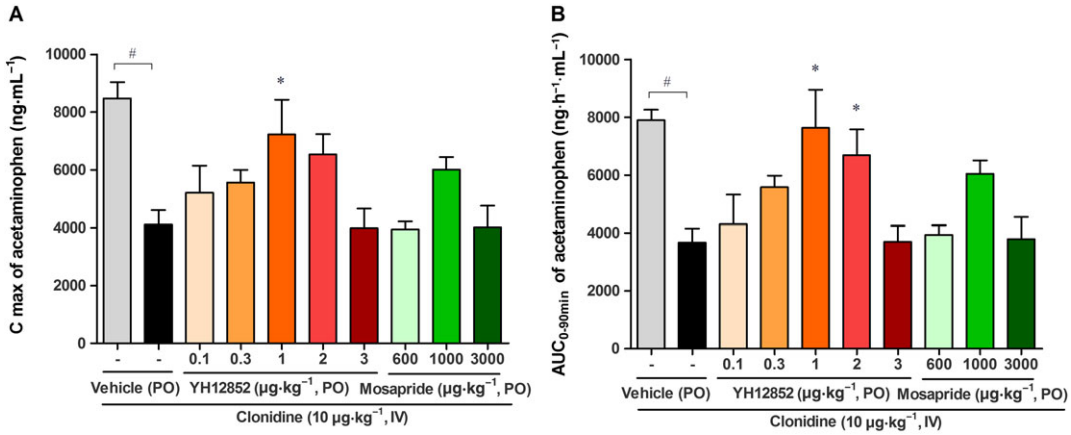

Figure 7

Effect of YH12852 on gastric emptying rate in dogs with clonidine-induced gastroparesis. (A) The Cmax of acetaminophen and (B) AUC of acetaminophen concentration were calculated as an indirect marker of gastric emptying rate, based on the fact that acetaminophen is not absorbed through the stomach but rapidly through the proximal small intestine. To induce gastroparesis, clonidine was administered i.v. 15 min before the acetaminophen solution: $n = 8$ for vehicle-treated group without clonidine injection, $n = 6$ for groups with treatment either of vehicle, YH12852 at 0.1 μg·kg^{-1} or mosapride at 600 or 3000 μg·kg^{-1} with clonidine injection and $n = 7$ for all the rest of the groups. Results are expressed as mean ± SEM and statistically analysed by one-way ANOVA followed by Dunnett's test (* $P < 0.05$ vs. the vehicle-treated control) or by Student's *t*-test (# $P < 0.05$, between two vehicle-treated groups with saline or clonidine administration).

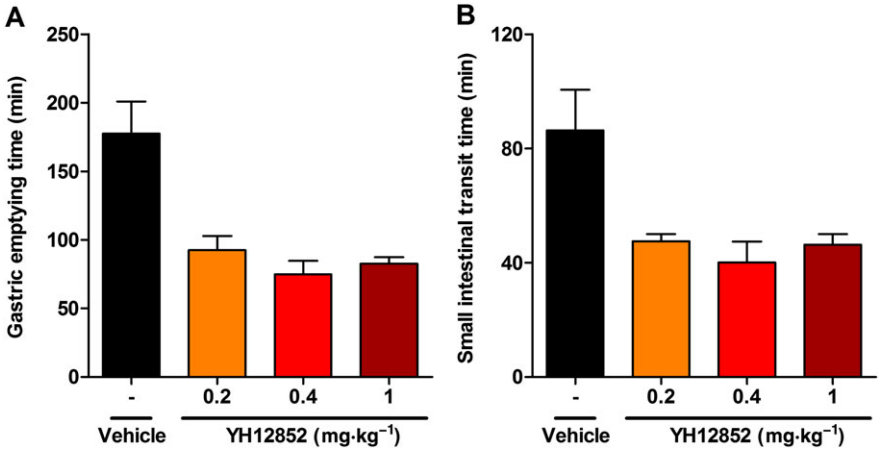

Figure 8

Effect of YH12852 on (A) gastric emptying and (B) small intestinal transit time in monkeys following single oral administration. For all groups, $n = 4$. Results are expressed as mean ± SEM, and statistical analysis was not carried out due to an insufficient number of animals used in the study.

over all the other targets tested. Unlike tegaserod or mosapride, YH12852 had no significant affinity for other 5-HT (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2A}$, 5-HT$_{2B}$ or 5-HT$_3$) and non 5-HT receptors or channels. It is now well established that the adverse cardiac effects of GI prokinetics are related to an interaction with molecular targets other than the 5-HT$_4$-receptors (Scarpignato, 2012). Consistent with the very low affinity of YH12852 towards the hERG channel (Table 2) contributing to a large safety margin (hERG IC$_{50}$/effective therapeutic plasma concentration > 13 800-fold), it had no meaningful effect on blood pressure, heart rate and electrocardiogram, as measured in male cynomolgus monkeys using a telemetry system after single p.o. administration of up to 60 mg·kg^{-1} of YH12852, and no significant QT prolongation was observed in human studies (ClinicalTrials.gov Identifier: NCT01870674). These findings suggest that the clinical use of YH12852 would be associated with a low cardiac safety risk.

The high selectivity of YH12852 combined with its highest *in vitro* affinity and potency and full agonistic activity at 5-HT$_4$ receptors was expected to translate into stronger *in vivo* potency than other 5-HT$_4$ agonists with less concern for reduced efficacy (e.g. mosapride). Indeed, p.o. administration of YH12852 (0.1–10 mg·kg^{-1}) significantly and

dose-dependently increased FPO in guinea pigs, and it was clearly superior to prucalopride with approximately 10-fold higher potency and a twofold higher maximal effect. In the same conditions, non-fasted conscious guinea pigs administered YH12852 (0.1–3 mg·kg^{-1}, p.o.) exhibited a significantly accelerated gastric emptying rate, showing it has therapeutic potential to treat gastroparesis, functional dyspepsia or both of them simultaneously (as the therapeutic ranges of YH12852 for upper and lower GI tract partly overlap). Furthermore, in conscious fasted beagle dogs with colonic motility monitoring sensors implanted, p.o. administration of YH12852 (3–30 μg·kg^{-1}) significantly and dose-dependently reduced the time to first GMC and also increased GMC frequency with statistical significance at 30 μg·kg^{-1}. The ED_{50} value of Y12852 on the time to first GMC was calculated as 4 μg·kg^{-1}, which is approximately 10 times more potent than prucalopride, based on the reported value of 40 μg·kg^{-1} in the literature (Briejer *et al.,* 2001). Oral administration of YH12852 at 1 μg·kg^{-1} significantly restored delayed gastric emptying to normal levels, while mosapride was less effective in improving gastric emptying than YH12852 and failed to reach statistical significance in a dog model of gastroparesis, even at 1000 μg·kg^{-1} (as the maximal effective dose). In a real-time fluoroscopy study using cynomolgus monkeys, oral administration of YH12852 (0.03–3 mg·kg^{-1}) markedly reduced both gastric emptying time and small intestinal transit time and was more effective than mosapride at the same dose of 1 mg·kg^{-1}.

In summary, YH12852 is a structurally novel and highly selective 5-HT_4 receptor agonist with excellent *in vivo* potency and robust efficacy in both the upper and lower GI tracts. Its overlapping therapeutic range in guinea pigs for upper and lower GI tracts suggests it has therapeutic potential to treat both upper and lower GI disturbance simultaneously. Tissue distribution analysis after oral administration showed that YH12852 was highly distributed to the GI tract as the target organ and not distributed into the brain. Based on its excellent pharmacological and pharmacokinetic profiles, YH12852 holds promise as a potential, next generation therapeutic agent to address the unmet needs of patients with functional GI motility disorders such as FC or gastroparesis and FC patients with gastroparesis.

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Author contributions

E.J.J., S.Y.C., H.N.H. and J.Y.S. were responsible for the study design, performing and monitoring experiments, data analysis and interpretation. E.J.J. was responsible for drafting the manuscript; E.J.J. and S.W.O. were responsible for manuscript revision; J.Y.S. contributed to data collection and supervision. All authors contributed to the critical interpretation of the data and revision of the manuscript.

Conflict of interest

All authors are employees of Yuhan Corporation and hold stock/share options in Yuhan Corporation.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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